Inoculation onto Solid Surfaces Protects *Salmonella* spp. during Acid Challenge: a Model Study Using Polyethersulfone Membranes

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Salmonellae are the most frequently reported cause of outbreaks of food-borne gastroenteritis in the United States. In clinical trials, the oral infective dose (ID) for healthy volunteers was estimated to be approximately 1 million cells. However, in reports from various outbreaks, the ID of *Salmonella* species associated with solid foods was estimated to be as few as 100 cells. We found that fresh-cut produce surfaces not only provided suitable solid support for pathogen attachment but also played a critical role in increasing the acid tolerance of the pathogen. However the acidic nature of certain produce played no role in making salmonellae resistant to stomach acidity. Inoculation onto fresh-cut produce surfaces, as well as onto inert surfaces, such as polyethersulfone membranes and tissue paper, increased the survival of salmonellae during acid challenge (50 mM Na-citrate, pH 3.0; 37°C; 2 h) by 4 to 5 log units. Acid challenge experiments using cells inoculated onto polyethersulfone membranes provided a model system suitable for studying the underlying fundamentals of the protection that occurs when *Salmonella* strains are associated with solid foods. The surface-associated acid protection, which was observed in several *Salmonella* strains, required de novo protein synthesis and was independent of stationary-phase sigma transcription factor.

The complex nature of today's food distribution chain has created opportunities for fresh-cut fruits and vegetables to become contaminated with pathogens (2, 5, 9). Moreover, different methods used to extend the shelf life of the fresh-cut produce are likely to pave the way for microbial growth and spoilage that may not occur under ordinary circumstances (6, 28). Growers have limited control over conditions in the field compared to those in an enclosed production facility. Prevention of contamination caused by human pathogens during the harvesting, washing, cutting, packaging, and transporting of the produce is of considerable importance to public health and, consequently, is a major issue for the fresh-cut-produce industry (8, 17, 23).

Salmonellae are the most frequently reported cause of food-borne gastroenteritis in the United States. Even though fresh fruits and vegetables are implicated less frequently than foods of animal origin, salmonellae have been isolated from several types of fruits and vegetables (3, 8, 10). Of the contaminated samples 44 out of 1,000 identified in a recent survey of high-volume imported fresh produce, about 80% (35 cases) were contaminated with *Salmonella* and 20% (9 cases) were contaminated with *Shigella* (http://vm.cfsan.fda.gov/~dms/prodsur6.htm). A number of consumer market research reports have predicted that the demand for fresh-cut fruits will increase rapidly in the near future, with food service establishments and school lunch programs being the major customers (1).

The number of bacteria consumed in contaminated food can vary considerably, as does the infective dose (ID) of enteric pathogens (7, 21). The ID of *Shigella flexneri*, which is commonly transmitted by person-to-person spread via a fecal-oral route, is approximately 100 cells (7). *Salmonella* spp. which primarily cause food-borne infections, are much less acid resistant, and their ID is still a controversial issue (7, 19, 22). In clinical trials where defined inocula were fed to human volunteers, the ID was estimated to be at least 10^6 bacteria (22). At the same time, there are reports from various outbreaks that when the cells were ingested with a food source, *Salmonella* spp. caused infection at a much lower ID ($\leq$100 cells) (11, 12, 18). It has been suggested that solid foods, especially those rich in fat, may protect salmonellae against stomach acidity (11, 29). The biochemical and molecular events leading to the surface-associated protection are not yet fully understood.

Under fasting conditions, the luminal pH in healthy volunteers is in the range of 1.5 to 5.5 (27). In this study, we demonstrate that various *Salmonella* strains that would normally be killed during acid challenge (pH 3.0; 37°C; 2 h), survived an identical acid challenge when inoculated on fresh-cut produce surfaces. The specific objective of the present study was to examine whether the surface inoculation affects the ability of salmonellae to survive acidic conditions that mimic those in the human stomach. Further, it was our intent to develop a model system which would enable us to design molecular biology experiments to study the phenomenon of the surface contact-mediated acid protection.

MATERIALS AND METHODS

Bacterial strains and media. *Salmonella enterica* serovar Typhimurium ATCC 14028s and its *rpoS* mutant strain SF1005 were obtained from Ferric Fang (13). Other *Salmonella* strains were obtained from the Salmonella Genetic Stock

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TABLE 1. *Salmonella* strains used in this study

Strain	Relevant genotype	Source or reference
Salmonella serovar Typhimurium		
14028s	Wild type	13
SF1005	Strain 14028s *rpoS*::Ap10	13
LT2	Natural isolate with *rpoS* mutation	SGSC
KK2051	LT2 *che*A501::Tn*10*	SGSC
KK2503	LT2 *fliC*::Tn*10*	SGSC
SJW1368	LT2 Δ(*cheW-CheA-motB-motA-flhC-flhD*)	SGSC
SJW3003	LT2 *motA1008*	SGSC
Salmonella serovar Enteritidis (SARB16)	Wild type	SGSC
Salmonella serovar Dublin (SARB12)	Wild type	SGSC
Salmonella serovar Paratyphi A (SARB42)	Wild type	SGSC
Salmonella serovar Paratyphi B (SARB43)	Wild type	SGSC
Salmonella serovar Pullorum (SARB51)	Wild type	SGSC
Salmonella serovar Pullorum (SARB52)	Wild type	SGSC
Salmonella serovar Anatum (SARB2)	Wild type	SGSC
Salmonella serovar Typhi (SARB64)	Wild type	SGSC

Center (SGSC), Calgary, Alberta, Canada (Table 1). Cultures were routinely started from freezer stocks for growth on Luria-Bertani (LB) medium. For some experiments, cells were grown in LB broth buffered with either 100 mM MES (morpholineethanesulfonic acid; pH 5.5) or 100 mM MOPS (morpholinepropanesulfonic acid; pH 8.0); these are referred to as adapted and unadapted growth conditions, respectively. Following overnight incubation at 37°C, a single colony was selected and inoculated into 10 ml of LB broth in a 125-ml Erlenmeyer flask. The cells were grown for 17 to 19 h at 37°C with shaking at 200 rpm to obtain stationary-phase cultures. Cultures from the stationary growth phase were harvested by centrifugation at 4,000 × *g* for 10 min, washed once with 3 volumes of saline (0.9% NaCl), and suspended in 350 to 400 μl of saline. The cells were allowed to incubate at room temperature for 15 to 20 min before acid challenge assays were performed.

Acid challenge assays. (i) Acid challenge in planktonic (free-cell suspension) state. Approximately 10^8 cells (5 μl of washed cell suspension) were suspended in 1 ml of 50 mM Na-citrate buffer (pH 3.0), which was equilibrated at 37°C. Acid challenge was performed for 2 h at 37°C. The cells were collected by centrifugation (12,000 × *g* for 10 min) and resuspended in 1.0 ml of 50 mM phosphate buffered saline (PBS; pH 7.2). Surviving cells were determined by estimating viable counts after appropriate dilutions made in 50 mM PBS or buffered peptone water (BPW) were plated at pH 7.3 on LB agar media. In addition, for treatments where a low survival rate was expected, a duplicate set of samples were processed in which cells (after acid challenge and centrifugation) were suspended in 100 μl of PBS before being plated on LB agar medium. Using this protocol, the appearance of a single colony at a starting cell density of 10^8 per ml during acid challenge sets the detection limit for the surviving population at 1.0×10^{-6}%.

(ii) Acid challenge of *S. enterica* cells inoculated onto solid surfaces. Granny Smith apples (*Malus domestica* Borkh), Roma tomatoes (*Lycopersicon esculentum* Mill.) and cucumber (*Cucumis sativus* L.) obtained from a local market were surface sterilized using 70% ethanol and sliced with a sterile knife. Approximately 2- to 3-mm-thick slices, weighing 8 to 12 g, were inoculated on the cut surface with bacterial cultures as described below. The pH at the surface of the fresh-cut produce was measured using pH paper indicator strips (Sigma Chemical Co., St. Louis, Mo.).

Acid challenge was performed on fresh-cut produce or on water agar (1.6% agar in distilled water) surfaces. Several replicates of the fruit slices (8 to 12 g each) were spotted with a *Salmonella* serovar Typhimurium cell suspension (5 μl each, containing 10^8 cells). The fruit slices were incubated in a closed container at room temperature for approximately 20 to 30 min in order to allow the cells to adhere to the sliced fruit surface. The cells on the fruit surface were acid challenged by homogenizing each slice in 100 ml of prewarmed (37°C) Na-citrate buffer (50 mM; pH 3.0) for 2 min at 230 rpm (Stomacher 400 circulator; Seward Inc., London, United Kingdom). There was no significant change in the pH (< 0.1 pH unit) upon homogenization. The homogenized produce was then incubated at 37°C for 2 h. After incubation, the fruit slices were homogenized again (230 rpm; 1 min), and serial dilutions in PBS were used for viable-count determinations on LB agar plates. To determine the recovery of the inoculated cells from the produce, an independent set was processed identically, except 100 ml of buffered peptone water (Difco Laboratories, Detroit, (Mich.) was used instead of Na-citrate buffer and viable counts were determined immediately. As controls, uninoculated fresh-cut produce slices were placed in BPW, homogenized as described above, and examined for the presence of contaminating bacteria.

Agar plates (1.6% agar [wt/vol] in distilled water) were prepared with approximately 20 ml of agar per plate. Four spots of *Salmonella* serovar Typhimurium cell suspension (5 μl each, containing 10^8 cells) were placed on the agar surface. The agar plates were incubated at room temperature for 20 to 30 min, after which the agar was peeled off the plates and processed as described for the fresh-cut produce.

(iii) Acid challenge of *Salmonella* serovar Typhimurium cells inoculated onto polyethersulfone membranes. Polyethersulfone membranes (0.22 μm thick) (Durapore membrane; Millipore Inc., Bedford, Mass.) were washed in distilled water for 1 h and dried at room temperature before they were laid on water agar plates (1.6% agar [wt/vol] in distilled water). *Salmonella* serovar Typhimurium cells (5 μl; approximately 10^8 cells) were placed on individual polyethersulfone membranes and allowed to incubate at room temperature for 20 to 30 min. To determine the optimum incubation time prior to acid challenge, cells were allowed to incubate on polyethersulfone membranes for 1 to 60 min. The cells resting on the polyethersulfone disk were acid challenged as described above. All the experiments were repeated at least three times. In some cases, chloramphenicol was added at a final concentration of 100 μg per ml for 20 min prior to inoculation onto membranes. The cells were then inoculated onto membranes, which were placed on water agar containing chloramphenicol (100 μg per ml). Acid challenge assays were performed as described above.

RESULTS

Survival of *Salmonella* serovar Typhimurium inoculated onto fresh-cut produce. The survival of *Salmonella* serovar Typhimurium under acidic conditions (pH 3.0) was tested while the cells were adhering to various fresh-cut fruits (Fig. 1). Our studies show that several fresh-cut fruits with a wide pH range allowed *Salmonella* serovar Typhimurium to survive acid challenge when the cells were inoculated on the surface. Neutral and inert support, such as water agar, also provided comparable protection during acid challenge. The same cells did not survive identical acid challenge in cell suspensions (planktonic state). Uninoculated fresh-cut produce was free of contaminating bacteria. Most of the inoculated cells of *Salmonella* serovar Typhimurium could be recovered from the fresh-cut produce surface in buffered peptone (cucumber, 93.1% ± 6.8%; tomato, 76.0% ± 15.4%; apple, 82.9% ± 12.0%) (Fig. 1).

Survival of *Salmonella* serovar Typhimurium inoculated onto inert supports. In order to determine if the nutrient composition of fresh-cut fruits had a role in providing protection against acid challenge, *Salmonella* serovar Typhimurium cells were inoculated on various inert supports, such as polyethersulfone membranes, Whatman filter paper, and tissue paper (low lint; nonabrasive) (Skillcraft, Duluth, Minn.) before the acid challenge assay (Fig. 2). When compared to the survival rate of planktonic cells, inoculation on various inert supports resulted in 4- to 5-log-fold better survival during acid challenge. The protection for Whatman filter paper and tissue
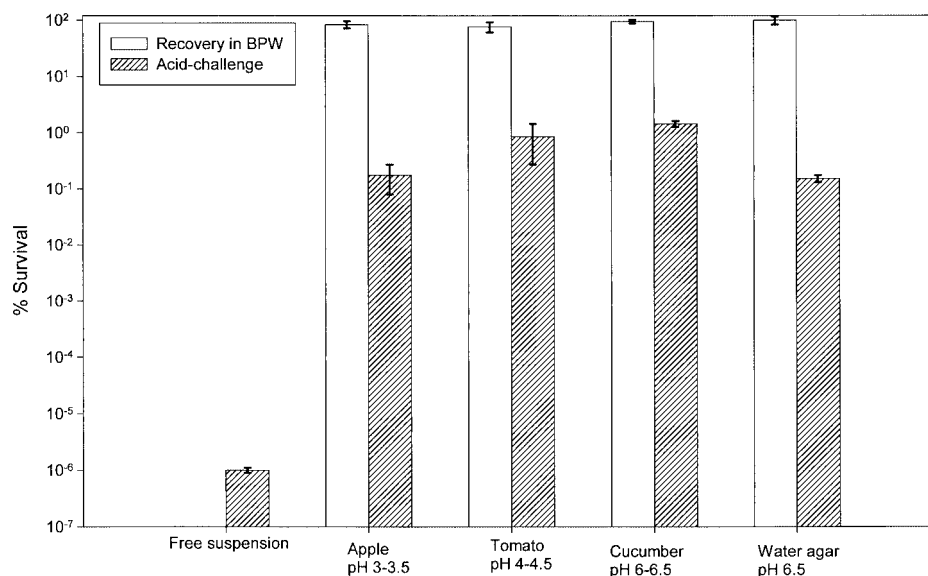
FIG. 1. Survival of acid-challenge by *Salmonella* serovar Typhimurium after inoculation onto various fresh-cut produce and water-agar surfaces. Recovery of inoculated cells from fresh-cut produce slices was estimated by extracting the produce in BPW (pH 7.5); acid challenge was performed by homogenizing the inoculated produce slices in 50 mM Na-citrate, pH 3.0, at 37°C for 2 h. The error bars indicate standard deviation.

paper was slightly lower than that observed for polyethersulfone membranes.

Surface contact-mediated acid protection requires de novo protein synthesis. Cells acquired the ability to survive acid challenge within the first 15 to 20 min after inoculation onto the polyethersulfone membrane (Fig. 3). The addition of a membrane disk into the assay tube containing planktonic cells at any point during the 2-h incubation did not protect the cells against acid challenge (data not shown). In order to determine if the initial incubation period of 15 to 20 min was due to the time necessary to synthesize new proteins, cells were treated

with sublethal concentrations of chloramphenicol during the first 20 min on polyethersulfone membranes before they were subjected to acid challenge (Table 2). The cells treated with chloramphenicol (100 μg/ml; 20 min) were viable but did not exhibit surface-associated high survival rates during acid challenge. The data support the concept that de novo protein synthesis is necessary after inoculation onto a solid support in order for the bacteria to survive subsequent acid challenge. Contact with solid surfaces per se was not required for higher
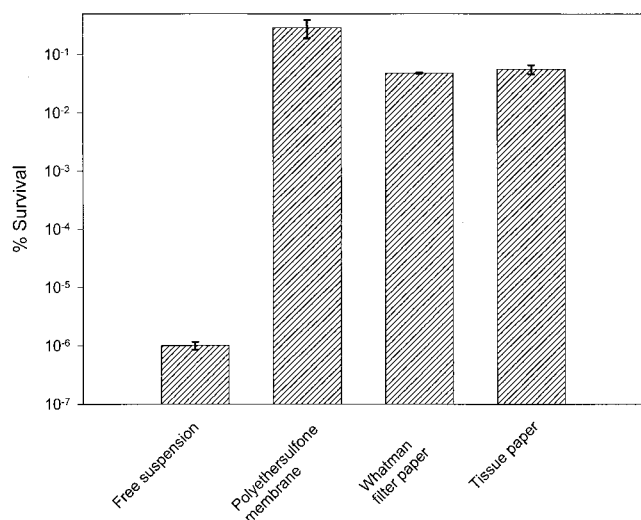

FIG. 2. Survival of acid challenge by *Salmonella* serovar Typhimurium after inoculation onto various solid supports. The error bars indicate standard deviation (not shown where smaller than the line thickness).
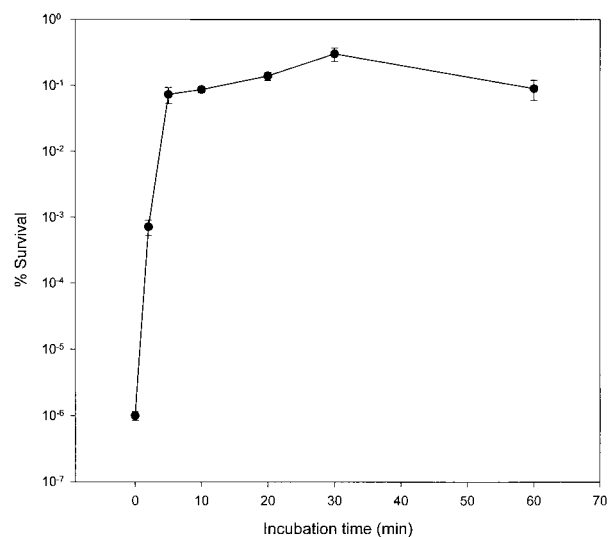

FIG. 3. Effect of incubation time on surface-associated acid tolerance acquired by *Salmonella* serovar Typhimurium on polyethersulfone membranes. The cells were placed on polyethersulfone membranes and incubated at room temperature for 0 to 60 min before acid challenge was initiated. The error bars indicate standard deviation (not shown where smaller than the symbol).

TABLE 2. Acid challenge response of planktonic and membrane-associated cells of *Salmonella* serovar Typhimurium

Type of cells used for acid challenge[a]	Pretreatment	% Cells recovered in saline[b]	% Cell survival after acid challenge
Planktonic	None	100	<0.00001
On polyethersulfone membrane	On water agar (room temperature; 20 min)	97.5 ± 8.5	0.30 ± 0.1
Planktonic	Chloramphenicol (100 μg/ml; 20 min)	95.0 ± 2.8	<0.00001
On polyethersulfone membrane	On water agar containing chloramphenicol (100 μg/ml; 20 min)	77.0 ± 11.3	0.05 ± 0.02
Planktonic	Inoculated on polyethersulfone membrane for 20 min and recovered in saline	81.5 ± 4.5	0.23 ± 0.1

[a] *Salmonella* serovar Typhimurium cells were acid challenged in either the planktonic state or after being placed on polyethersulfone membranes for 20 min.
[b] *Salmonella* serovar Typhimurium cells inoculated on polyethersulfone membranes were recovered by being vortexed in saline as a control to determine the percent cell recovery.

survival during acid challenge, as cells dissociated in saline after being in contact with polyethersulfone membranes for 20 min were able to exhibit high survival rates even when acid challenged in the planktonic state (Table 2). Our initial attempts to identify the newly synthesized protein(s) by one-dimensional polyacrylamide gel electrophoresis were inconclusive (data not shown).

Surface contact-mediated protection and cell density during acid challenge. To determine if the inoculum size had any effect on the bacterial survival rate and/or the ability to recover cells from polyethersulfone membranes in saline, serial dilutions of *Salmonella* serovar Typhimurium were inoculated onto polyethersulfone membranes and acid challenged as described above (Fig. 4). Identical sets of filters were extracted with saline to determine the effect of cell density on the recovery of cells from polyethersulfone membranes. Despite variations in the inoculum size (10^8 to 10^4 cells), cell recovery and the survival rate of bacteria inoculated onto polyethersulfone membranes were fairly constant.

Surface contact-mediated acid protection in other strains of *Salmonella*. In order to determine if the phenomenon described above is restricted to *Salmonella* serovar Typhimurium or is applicable to other salmonellae, several strains of *Salmonella* spp. were examined for survival of acid challenge after their inoculation onto polyethersulfone membranes (Table 3). Our data indicate that a number of *Salmonella* strains, such as *S. enterica* serovar Enteritidis, *S. enterica* serovar Dublin, *S. enterica* serovar Paratyphi B, *S. enterica* serovar Anatum, and *S. enterica* serovar Typhi, showed severalfold-higher survival rates when they were acid challenged after inoculation onto polyethersulfone membranes. However, no such protection was observed for *S. enterica* serovar Paratyphi A and *S. enterica* serovar Pullorum strains. *Salmonella* serovar Typhimurium LT2 is a natural isolate carrying an *rpoS* mutation. In order to determine if the protection observed with surface-inoculated cells is dependent on the *rpoS* regulon, the survival of strain LT2 was compared with that of strain SF1005. Both strains exhibited comparable acid survival before and after inoculation onto polyethersulfone membranes, suggesting that the increased acid protection observed subsequent to inoculation onto solid support occurs independently of *rpoS* regulation.

Effect of culture conditions on surface contact-mediated acid protection. *Salmonella* serovar Typhimurium synthesizes several proteins crucial for acid survival during its stationary
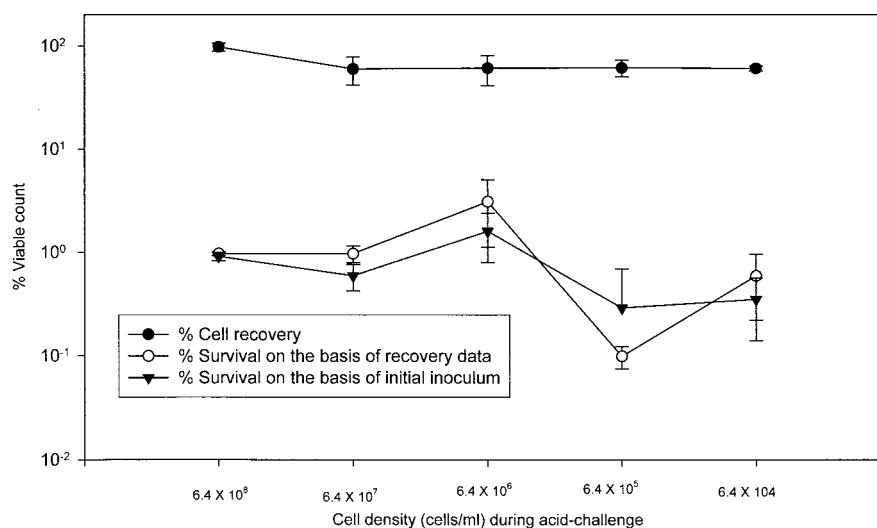

FIG. 4. Effect of inoculum size on surface-associated acid tolerance acquired by *Salmonella* serovar Typhimurium on polyethersulfone membranes. Various numbers of cells were placed on polyethersulfone membranes, and after 20 min of incubation at room temperature, the cells were subjected to acid challenge. An identical set of filters was extracted in saline to determine the effect of the inoculum size on the recovery of cells. The percentage of cells surviving the acid challenge was computed based on either cells recovered in saline or the initial inoculum. The error bars indicate standard deviation (not shown where smaller than the line thickness).

TABLE 3. Survival of acid challenge by various *Salmonella* strains and chemotaxis, flagellum and motility mutants in the planktonic versus surface-associated states

Strain	% Survival after acid challenge	
	Planktonic cells	On polyethersulfone membrane
Salmonella *serovar* Typhimurium		
14028s	≤0.000001	0.3 ± 0.07
LT2	≤0.000001	0.02 ± 0.01
KK2051	≤0.000001	0.14 ± 0.05
KK2503	≤0.000001	0.49 ± 0.3
SJW1368	≤0.000001	0.18 ± 0.09
SJW3003	≤0.000001	0.15 ± 0.07
Salmonella serovar Enteritidis (SARB16)	0.00004 ± 0.000004	0.89 ± 0.01
Salmonella serovar Dublin (SARB12)	≤0.000001	0.39 ± 0.05
Salmonella serovar Paratyphi A (SARB42)	≤0.000001	≤0.000001
Salmonella serovar Paratyphi B (SARB43)	≤0.000001	1.2 ± 0.8
Salmonella serovar Pullorum (SARB51)	≤0.000001	≤0.000001
Salmonella serovar Pullorum (SARB52)	≤0.000001	≤0.000001
Salmonella serovar Anatum (SARB2)	≤0.000001	0.43 ± 0.32
Salmonella serovar Typhi (SARB64)	≤0.000001	0.32 ± 0.20

growth phase (14, 15). From this large set of proteins, the synthesis of about 50 proteins has been demonstrated to be under the control of the *rpoS* regulon (16, 24). *Salmonella* serovar Typhimurium also has an alternative acid tolerance pathway which requires growth under acidic conditions and is

also induced in the stationary growth phase (4). Growth under acidic (pH 5.5) and alkaline (pH 8.0) conditions in LB medium allowed us to compare the contributions of the two acid protection pathways to the increased acid survival of cells inoculated on solid supports (Fig. 5). Wild-type cells from unadapted growth conditions (LB-MOPS; pH 8.0) (i.e., the *rpoS* pathway is active) were more sensitive to acid challenge on polyethersulfone membranes than adapted cultures (LB-MES; pH 5.5) (i.e., the *rpoS*-independent pathway). Likewise, strain SF1005 survived acid challenge better when grown at pH 5.5, indicating that cells probably utilize an *rpoS*-independent acid survival pathway when inoculated onto solid supports.

Role of motility and flagella in surface-mediated acid protection. We tested several mutants of *Salmonella* serovar Typhimurium, defective in motility and flagellum synthesis, for acid survival after they were inoculated on solid supports (Table 3). All the mutants were able to survive the acid challenge at a higher rate after their inoculation onto polyethersulfone membranes, indicating that cells may be utilizing alternate adherence mechanisms other than those involving flagella.

DISCUSSION

The studies presented here were undertaken to examine the acid challenge response of *Salmonella* serovar Typhimurium cells subsequent to their inoculation onto solid surfaces. An inert support, i.e., a polyethersulfone membrane, was selected (150 to 200 μm thick, low protein binding, and high wettability) to facilitate the acquisition of data. After 20 to 30 min of incubation on polyethersulfone membranes, most of the *Salmonella* serovar Typhimurium cells (97.5% ± 8.5%) were recovered by vortexing the membranes in either saline or PBS (Table 2). Thus, the association of cells with polyethersulfone
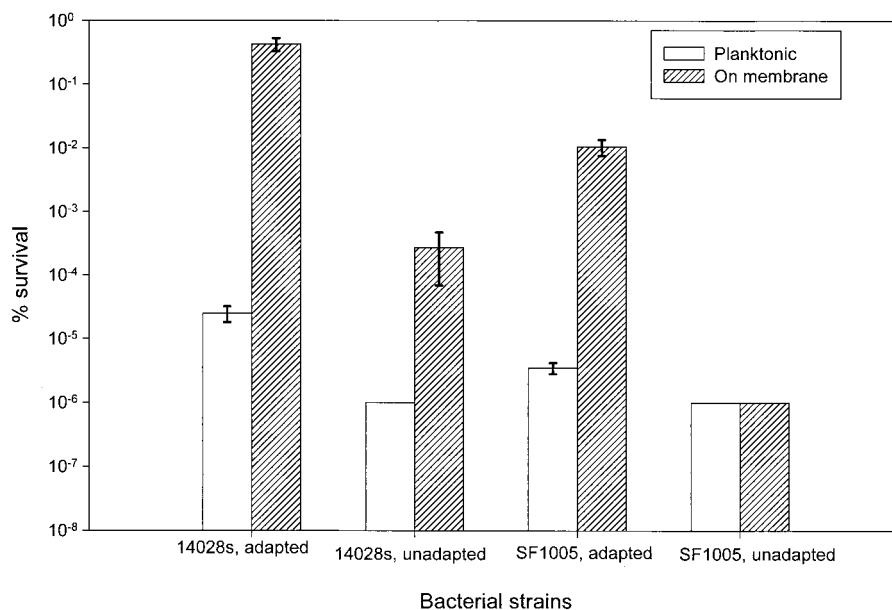

FIG. 5. Effect of pH of growth medium and *rpoS* genotype on surface-associated acid tolerance of *Salmonella* serovar Typhimurium. The wild-type (14028s) and *rpoS* mutant (SF1005) cells were grown under adaptive (LB-MES; pH 5.5) or unadaptive (LB-MOPS; pH 8.0) growth conditions and were acid challenged either after inoculation onto polyethersulfone membranes or in the planktonic state. The error bars indicate standard deviation (not shown where smaller than the line thickness).

membranes appears to be loose and certainly not at the stage where it could be referred to as a biofilm. The degree of protection during acid challenge as a result of inoculation onto polyethersulfone membranes was 4 to 5 log-fold. In the event of pathogen outbreaks associated with solid foods, higher protection may be observed, as certain foods, in addition to providing a solid surface for attachment, may also be a source of essential amino acids, such as glutamate, lysine, or arginine (10a, 24, 29). The experiments described here provide a system suitable for studying the underlying fundamentals of protection observed when *Salmonella* strains are associated with solid foods (11, 12, 18). Previous studies have indicated that once the pH barrier of the stomach has been breached, the number of surviving bacteria reaching the intestines does not affect the severity of the disease (26, 27). This further implies that the ability to adhere to solid surfaces may significantly influence the ID of a specific enteric pathogen, and it needs to be analyzed further.

It has been suggested that *Salmonella* outbreaks with a low ID are often associated with a food source with a high fat content (11, 12, 18). Boiled egg white (but not boiled rice), which is low in fat but rich in proteins, is also reported to protect *Salmonella* from acid challenge (29). Based on the data presented here, it appears that the primary factor in the observed protection by solid foods could be merely the availability of a solid support for pathogen attachment. While the precise roles played by either the fat or protein content of the food in acid protection of bacteria is yet to be determined, it appears that the ability of ground beef to raise the pH of the acidified medium did play a crucial role (29). Placing a polyethersulfone membrane or a fruit slice in the acid challenge buffer solution did not cause any change in pH during the 2-h incubation period (<0.1 pH unit) (data not shown). In fact, acidity at the produce surface had little influence on the induction of an acid tolerance response or on surface contact-mediated protection (Fig. 1). The observed protection appeared widespread across several strains of *Salmonella* species and with many different neutral solid supports, such as ordinary tissue paper and Whatman filter paper (Fig. 2). The lack of surface-associated protection during acid challenge in *Salmonella* serovar Pullorum and *Salmonella* serovar Paratyphi A is intriguing. Whether it reflects true genetic variation in acid survival mechanisms among different strains will require further investigation.

Salmonella serovar Typhimurium possesses at least two stationary-phase acid tolerance systems, one that is acid induced and σ transcription factor (encoded by *rpoS*) independent and another that is unresponsive to pH but is regulated by the σ transcription factor (4, 20). Our data indicate that surface contact-mediated protection is more robust when cells are grown under mild acidic conditions (LB-MES; pH 5.5). The contribution of an *rpoS*-mediated acid survival pathway appears minimal, since strain SF1005 was able to protect itself once inoculated onto solid surfaces, provided the cells were grown in LB-MES medium (pH 5.5) (Fig. 5). The acid-inducible *rpoS*-independent acid tolerance system has recently been characterized in *Salmonella* serovar Typhimurium, in which the outer membrane protein, OmpR, appears to play a crucial role (4, 20). The exact biochemical mechanism by which OmpR functions is not yet known. It is interesting to note that the acid-inducible OmpR system has been speculated to be a major virulence factor of the organism (4, 16, 24).

In *E. coli*, chemotaxis and type I pilus genes (*cheA* and *fimH*, respectively) play a critical role in attachment and biofilm formation (25, 30). Although the identity of the de novo-synthesized proteins is not yet clear, the proteins do not appear to be the products of the *che* (chemotaxis), *mot* (motility), or *flg* (flagella) operon (Table 3). *Salmonella* serovar Typhimurium cells required 15 to 20 min to acquire contact-mediated acid protection after their inoculation onto polyethersulfone membranes (Fig. 3). No surface contact-mediated acid protection was observed in the presence of chloramphenicol (Table 2). This indicates that synthesis of new proteins might be induced in the cells due to the surface contact with polyethersulfone membranes. Genomewide analysis using RNA transcripts obtained from the surface-associated cells may help to identify likely transcriptional or translational regulatory elements or new genes which may have roles in acid protection.

REFERENCES

1. **Anonymous.** 2000. Fresh sliced apples: waiting to boom? Fresh Cut **June:** 18–22.
2. **Anonymous.** 1998. National advisory committee on the microbiological criteria for foods: microbiological safety evaluations and recommendations on fresh produce. Food Control **9:**321–347.
3. **Backer, H. D., J. C. Mohle-Boetani, S. B. Werner, S. L. Abbott, J. Farrar, and D. J. Vugia.** 2000. High incidence of extra-intestinal infections in a *Salmonella havana* outbreak associated with alfalfa sprouts. Public Health Rep. **115:**339–345.
4. **Bang, I. S., B. H. Kim, J. W. Foster, and Y. K. Park.** 2000. OmpR regulates the stationary-phase acid tolerance response of *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **182:**2245–2252.
5. **Beuchat, L. R.** 1996. Pathogenic microorganisms associated with fresh produce. J. Food Prot. **59:**204–216.
6. **Beuchat, L. R., and J. H. Ryu.** 1997. Produce handling and processing practices. Emerg. Infect. Dis. **3:**459–465.
7. **Blaser, M. J., and L. S. Newman.** 1982. A review of human *Salmonellosis*. I. Infective dose. Rev. Infect. Dis. **4:**1096–1106.
8. **Brackett, R. E.** 1999. Incidence, contributing factors, and control of bacterial pathogens in produce. Postharvest Biol. Technol. **15:**301–311.
9. **Brackett, R. E.** 1994. Microbiological spoilage and pathogens in minimally processed fruits and vegetables, p. 269–312. *In* R. C. Wiley (ed.), Minimally processed refrigerated fruits and vegetables. Van Nostrand Reinhold, New York, N.Y.
10. **Campbell, J. V., J. Mohle-Boetani, R. Reporter, S. Abbott, J. Farrar, M. Brandl, R. Mandrell, and S. B. Werner.** 2001. An outbreak of *Salmonella* serotype Thompson associated with fresh cilantro. J. Infect. Dis. **183:**984–987.
10a. **Cui, S., J. Meng, and A. A. Bhagwat.** 2001. Availability of glutamate and arginine during acid challenge determines cell density-dependent survival phenotype of *Escherichia coli* strains. Appl. Environ. Microbiol. **67:**4914–4918.
11. **D'Aoust, J. Y.** 1985. Infective dose of *Salmonella typhimurium* in cheddar cheese. Am. J. Epidemiol. **122:**717–719.
12. **D'Aoust, J. Y., B. J. Aris, P. Thisdele, A. Durante, N. Brisson, D. Dragon, G. Leachapelle, M. Johnston, and R. Laidley.** 1975. *Salmonella eastbourne* outbreak associated with chocolate. Can. Inst. Food. Sci. Technol. J. **8:**181–184.
13. **Fang, F. C., S. J. Libby, N. A. Buchmeier, P. C. Loewen, J. Switala, J. Harwood, and D. G. Guiney.** 1992. The alternative sigma factor katF (rpoS) regulates *Salmonella* virulence. Proc. Natl. Acad. Sci. USA **89:**11978–11982.
14. **Foster, J. W.** 1991. *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. J. Bacteriol. **173:**6896–6902.
15. **Foster, J. W., and H. K. Hall.** 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. J. Bacteriol. **172:**771–778.
16. **Foster, J. W., and M. Moreno.** 1999. Inducible acid tolerance mechanisms in enteric bacteria. Novartis Found. Symp. **221:**55–69.
17. **Hao, Y. Y., and R. E. Brackett.** 1993. Influence of modified atmosphere on growth of vegetable spoilage bacteria in media. J. Food Prot. **56:**223–228.

18. **Hedberg, C. W., J. A. Korlath, J. Y. D'Aoust, K. E. White, W. L. Schell, M. R. Miller, D. N. Cameron, K. L. MacDonald, and M. T. Osterholm.** 1992. A multistate outbreak of *Salmonella javiana* and *Salmonella oranienburg* infections due to consumption of contaminated cheese. JAMA **268:**3203–3240.

19. **Hornick, R. B., S. E. Greisman, T. E. Woodward, H. L. DuPont, A. T. Dawkins, and M. J. Snyder.** 1970. Typhoid fever: pathogenesis and immunological control. N. Engl. J. Med. **283:**739–746.

20. **Lin, J., I. S. Lee, J. Frey, J. L. Slonczewski, and J. W. Foster.** 1995. Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*. J. Bacteriol. **177:**4097–4104.

21. **Lin, J., M. P. Smith, K. C. Chapin, H. S. Baik, G. N. Bennett, and J. W. Foster.** 1996. Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. Appl. Environ. Microbiol. **62:**3094–3100.

22. **McCullough, N. B., and C. W. Eisele.** 1951. Experimental human salmonellosis. I. Pathogenicity of strains of *Salmonella meleagridis* and *Salmonella anatum* obtained from spray-dried whole egg. J. Infect. Dis. **88:**278–289.

23. **Odumeru, J. A., S. J. Mitchell, D. M. Alves, J. A. Lynch, A. J. Yee, S. L. Wang, S. Styliadis, and J. M. Farber.** 1997. Assessment of the microbiological quality of ready-to-use vegetables for health-care food services. J. Food Prot. **60:**954–960.

24. **Park, Y. K., B. Bearson, S. H. Bang, I. S. Bang, and J. W. Foster.** 1996. Internal pH crisis, lysine decarboxylase and the acid tolerance response of *Salmonella typhimurium*. Mol. Microbiol. **20:**605–611.

25. **Pratt, L. A., and R. Kolter.** 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. **30:**285–293.

26. **Schlech, W. F., D. P. Chase, and A. Badley.** 1993. A model of food-borne *Listeria monocytogenes* infection in the Sprague-Dawley rat using gastric inoculation: development and effect of gastric acidity on infective dose. Int. J. Food Microbiol. **18:**15–24.

27. **Verdu, E., F. Viani, D. Armstrong, R. Fraser, H. H. Siegrist, B. Pignatelli, J.-P. Idstrom, C. Lederberg, A. L. Blum, and M. Fried.** 1994. Effect of omeprazole on intragastric bacterial counts, nitrates, nitrites, and N-nitroso compounds. Gut **35:**455–460.

28. **Watada, A. E.** 1997. Quality maintainance of fresh-cut fruits and vegetables. Foods Biotechnol. **6:**229–233.

29. **Waterman, S. R., and P. L. C. Small.** 1998. Acid-sensitive enteric pathogens are protected from killing under extremely acidic conditions of pH 2.5 when they are inoculated onto certain solid food sources. Appl. Environ. Microbiol. **64:**3882–3886.

30. **Watnick, P., and R. Kolter.** 2000. Biofilm, city of microbes. J. Bacteriol. **182:**2675–2679.